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Neopestalotiopsis rosicola sp. nov. causing stem canker of *Rosa chinensis* in China

Ning Jiang[1], Guido Bonthond[2], Xin-lei Fan[1], Cheng-ming Tian[1]*

[1] *The Key Laboratory for Silviculture and Conservation of Ministry of Education,*
Beijing Forestry University, Qinghua Eastern Road 35, Haidian District, Beijing, China
[2] *Westerdijk Fungal Biodiversity Institute,*
Uppsalalaan 8, 3584 CT Utrecht, The Netherlands

* Correspondence to: *chengmt@bjfu.edu.cn*

Abstract—Two specimens of *Neopestalotiopsis* were collected from China rose in Jiangsu Province, China. Cankers and conidiomata were formed on stems of the infected hosts. Phylogenetic analysis of the combined ITS, tef1, and tub2 DNA markers revealed that both strains were conspecific and different from all other described *Neopestalotiopsis* species. The two strains are described and illustrated here as a new species, *Neopestalotiopsis rosicola*, and compared with related species genetically, morphologically and in host association. This is the first report of *Neopestalotiopsis* species causing China rose stem canker in China.

Key words —morphology, *Sporocadaceae*, phylogeny, taxonomy

Introduction

Roses represent one of the most important groups of horticultural and ornamental plants throughout the world (Krüssmann 1981). China rose (*Rosa chinensis*) is cultivated widely in cities as an ornamental flower and used in traditional Chinese medicine (Han & al. 2017). Disease management in large scale rose-producing plantations is generally effective in preventing plant infection; however, during our investigation we discovered roses killed by fungal pathogens.

Species of *Neopestalotiopsis* (*Sporocadaceae;* Jaklitsch & al. 2016, Hongsanan & al. 2017), many previously treated as *Pestalotiopsis,* include important plant pathogens (Maharachchikumbura & al. 2013, Jayawardena

& al. 2015) and plant endophytes (Watanabe & al. 2010, Debbab & al. 2013). Maharachchikumbura & al. (2014) split *Pestalotiopsis* sensu lato into three genera—*Neopestalotiopsis*, *Pseudopestalotiopsis*, *Pestalotiopsis* sensu stricto—based on morphology and phylogeny. *Neopestalotiopsis* with the generic type *N. protearum* (Crous & L. Swart) Maharachch. & al. could be distinguished morphologically from *Pseudopestalotiopsis* and *Pestalotiopsis* sensu stricto by its versicolorous median cells and conidiophores often reduced to conidiogenous cells (Maharachchikumbura & al. 2014) and molecularly by *Neopestalotiopsis* forming a distinct clade in the LSU phylogeny (Maharachchikumbura & al. 2014). Various *Neopestalotiopsis* species are known to cause diseases, e.g., *N. vitis* Jayaward. & al. causing a leaf spot disease of grapevine (Jayawardena & al. 2016). Based on morphological and phylogenetic analyses of ITS, TEF1, and TUB2, we describe a new *Neopestalotiopsis* species causing a disease of *Rosa chinensis* in southern China.

Materials & methods

Samples and isolates

Fresh *Neopestalotiopsis* specimens were collected from infected stems or twigs on *Rosa chinensis* in Jiangsu Province, China. Single conidial isolates were obtained from fruiting bodies by removing a mucoid conidial mass from pycnidial ostioles following a modified method of Fan & al. (2014), spreading the suspension on the surface of 1.8% potato dextrose agar (PDA), and incubating at 25 °C for up to 24 h. Single germinating conidia were removed and plated onto fresh potato dextrose agar (PDA). Two strains were used in the phylogenetic analysis (TABLE 1). Specimens and isolates of the new species are deposited in the Museum of Beijing Forestry University, Beijing, China (BJFC). Axenic cultures are maintained in the China Forestry Culture Collection Center, Beijing, China (CFCC).

Morphological studies

Fruiting bodies were observed morphologically on diseased plant tissues and in culture following Maharachchikumbura & al. (2012). Fifty spores were selected randomly for measurement using a Leica DM 2500compound light microscope. Colony characteristics observed and recorded from cultures incubated on PDA in the dark at 25°C at 3, 7, and 15 days included colony color, texture, and the arrangement of the conidiomata.

Pathogenicity test

Healthy China rose stems (*Rosa chinensis*) were surface sterilized according to Feng & al. (2014). Considering China roses are planted in cities and are pruned, we applied a wound on stems of the treatment and control groups and covered the wound either with mycelium or with pure PDA. The inoculated stems were wrapped with a plastic film and incubated at 25 °C for 10 days.

DNA extraction, PCR amplification, and sequencing

Genomic DNA was extracted from 7-day old colonies grown on PDA with cellophane using a modified CTAB method (Doyle & Doyle 1990). DNA fragment size was checked with electrophoresis on 1% agarose gels, and the concentration was measured using a Thermo NanoDrop™ 2000 according to the user manual (Desjardins & al. 2009).

For the amplification of ITS, TEF1, and TUB2, we employed a PTC-200 DNA Engine Peltier Thermal Cycler and primer pairs ITS5/ITS4 (White & al. 1990), BT2A/BT2B (Glass & Donaldson 1995, O'Donnell & Cigelnik 1997), 728F/EF2 (O'Donnell & Cigelnik 1997, Carbone & Kohn 1999). The PCR amplification products were inspected visually by electrophoresis in 2% agarose gels. DNA was sequenced using an ABI PRISM® 3730XL DNA Analyzer with BigDye® Terminator Kit v.3.1 at the Shanghai Invitrogen Biological Technology Company Limited in Beijing.

DNA sequence analysis

Our sequences and reference sequences obtained from GenBank (TABLE 1) were aligned using MAFFT v.6 (Katoh & Toh 2010) and edited manually using MEGA6 (Tamura & al. 2013). The alignments were concatenated for phylogenetic analyses. Maximum parsimony (MP) analysis was conducted with PAUP v.4.0b10 (Swofford 2003), maximum likelihood (ML) analysis with PhyML v.7.2.8 (Guindon & al. 2010), and Bayesian inference (BI) with MrBayes v.3.1.2 (Ronquist & Huelsenbeck 2003). The phylogenetic tree included our new ITS, TEF1, and TUB2 sequences and sequences from ex-type specimens in recent studies (Maharachchikumbura & al. 2014, Jayawardena & al. 2016).

Pseudopestalotiopsis cocos (CBS 272.29) and *Pestalotiopsis trachicarpicola* (OP 068) were selected as the outgroup in this analysis. Trees were visualized and examined using FigTree v.1.3.1 (Rambaut & Drummond 2010).

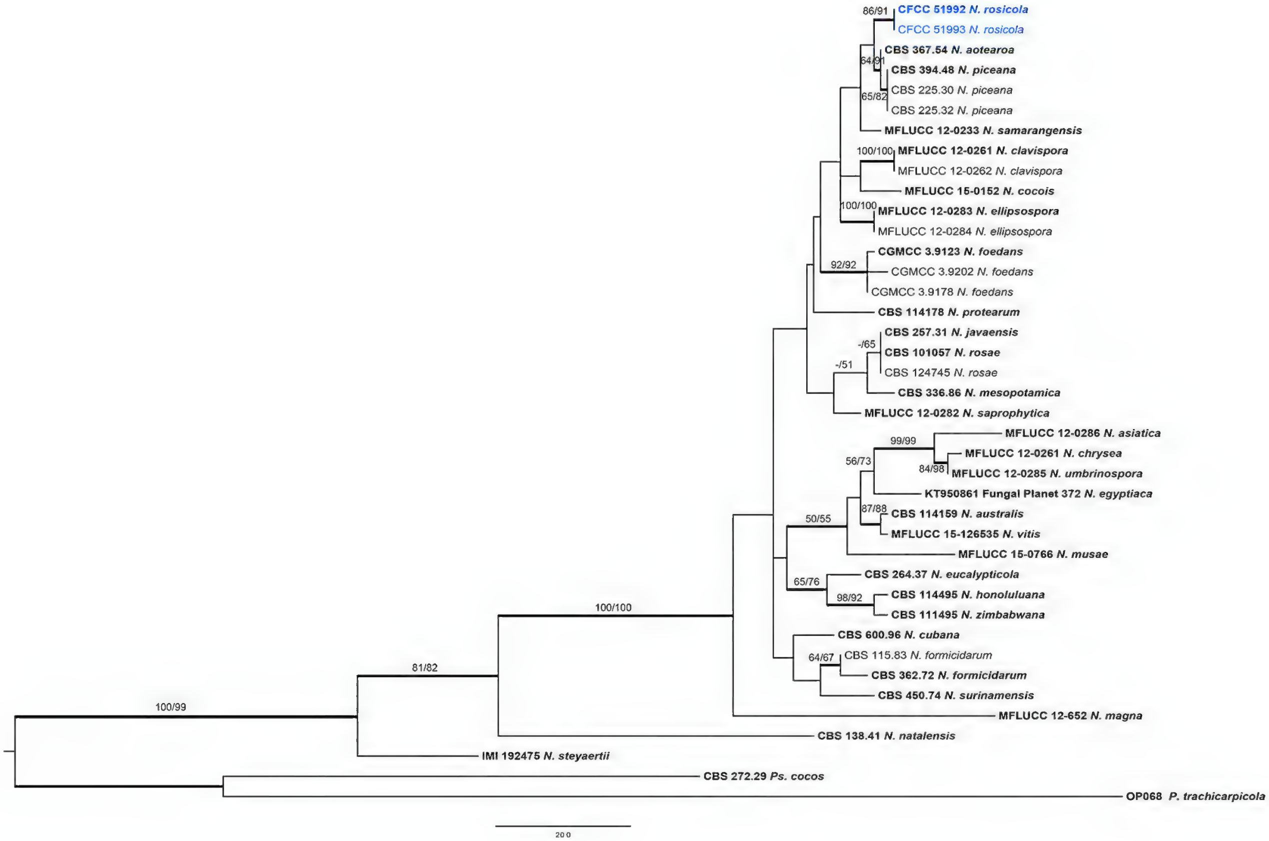
MP analysis was performed by a heuristic search option of 1000 random-addition sequences with a tree bisection and reconnection (TBR) algorithm. Maxtrees were set to 5000, branches of zero length were collapsed and all equally parsimonious trees were saved. Other calculated parsimony scores were tree length (TL), consistency index (CI), retention index (RI) and rescaled consistency (RC). ML analysis was performed with a GTR substitution model (Guindon & al. 2010). The branch support was evaluated with a bootstrapping (BS) method of 1000 replicates (Hillis & Bull 1993). For the BI analysis, a Markov Chain Monte Carlo (MCMC) algorithm was performed (Rannala & Yang 1996). The optimal substitution model for each locus was determined with MrModeltest v.2.3 (Posada & Crandall 1998). Sequence data were deposited in GenBank (TABLE 1). The multilocus alignment file was deposited in TreeBASE (www.treebase.org) as accession 20559. The nomenclatural novelty was deposited in MycoBank (Crous & al. 2004).

Table 1. Sequences of *Neopestalotiopsis*, *Pestalotiopsis*, and *Pseudopestalotiopsis* used in the molecular analysis

Species	Isolate	Host / Substrate	Location	GenBank		
				ITS	tef1	tub2
N. aotearoa	CBS 367.54	Canvas	New Zealand	KM199369	KM199526	KM199454
N. asiatica	MFLUCC 12-0286	Unidentified tree	China	JX398983	JX399049	JX399018
N. australis	CBS 114159	*Telopea* sp.	Australia	KM199348	KM199537	KM199432
N. chrysea	MFLUCC 12-0261	Dead leaves	China	JX398985	JX399051	JX399020
N. clavispora	MFLUCC 12-0281*	*Magnolia* sp.	China	JX398979	JX399045	JX399014
	MFLUCC 12-0280	*Magnolia* sp.	China	JX398978	JX399044	JX399013
N. cocois	MFLUCC 15-0152	—	—	KX789687	KX789689	—
N. cubana	CBS 600.96	Leaf litter	Cuba	KM199347	KM199521	KM199438
N. egyptiaca	CBS 140162	*Mangifera indica*	Egypt	KP943747	KP943748	KP943746
N. ellipsospora	MFLUCC 12-0283*	Dead plant material	China	JX398980	JX399047	JX399016
	MFLUCC 12-0284	Dead plant material	Thailand	JX398981	JX399046	JX399015
N. eucalypticola	CBS 264.37	*Eucalyptus globulus*	—	KM199376	KM199551	KM199431
N. foedans	CGMCC 3.9123*	Mangrove plant	China	JX398987	JX399053	JX399022
	CGMCC 3.9178	*Neodypsis decaryi*	China	JX398989	JX399055	JX399024
	CGMCC 3.9202	*Calliandra haematocephala*	China	JX398988	JX399054	JX399023
N. formicidarum	CBS 362.72*	Dead *Formicidae*	Ghana	KM199344	KM199519	KM199444
	CBS 115.83	Plant debris	—	KM199358	KM199517	KM199455
N. honoluluana	CBS 114495	*Telopea* sp.	USA: Hawaii	KM199364	KM199548	KM199457
N. javaensis	CBS 257.31	*Cocos nucifera*	Indonesia	KM199357	KM199543	KM199437
N. magna	MFLUCC 12-652	*Pteridium* sp.	France	KF582795	KF582791	KF582793

Species	Isolate	Host / Substrate	Location	GenBank		
				ITS	tef1	tub2
N. mesopotamica	CBS 336.86	*Pinus brutia*	Iraq	KM199362	KM199555	KM199441
N. musae	MFLUCC 15-0776	—	—	KX789683	KX789685	KX789686
N. natalensis	CBS 138.41	*Acacia mollissima*	South Africa	KM199377	KM199552	KM199466
N. piceana	CBS 394.48*	*Picea* sp.	UK	KM199368	KM199527	KM199453
	CBS 254.32	*Cocos nucifera*	Indonesia	KM199372	KM199529	KM199452
	CBS 225.30	*Mangifera indica*	—	KM199371	KM199535	KM199451
N. protearum	CBS 114178	*Leucospermum cuneiforme*	Zimbabwe	JN712498	KM199542	KM199463
N. rosae	CBS 101057*	*Rosa* sp.	New Zealand	KM199359	KM199523	KM199429
	CBS 124745	*Paeonia suffruticosa*	USA	KM199360	KM199524	KM199430
N. rosicola	**CFCC 51992***	***Rosa chinensis***	**China**	**KY885239**	**KY885243**	**KY885245**
	CFCC 51993	***Rosa chinensis***	**China**	**KY885240**	**KY885244**	**KY885246**
N. samarangensis	MFLUCC 12-0233	*Syzygium samarangense*	Thailand	JQ968609	JQ968611	JQ968610
N. saprophytica	MFLUCC 12-0282	*Magnolia* sp.	China	KM199345	KM199538	KM199433
N. steyaertii	IMI 192475	*Eucalyptus viminalis*	Australia	KF582796	KF582792	KF582794
N. surinamensis	CBS 450.74	Soil under *Elaeis guineensis*	Suriname	KM199351	KM199518	KM199465
N. umbrinospora	MFLUCC 12-0285	Unidentified plant	China	JX398984	JX399050	JX399019
N. vitis	MFLUCC 15-1265	*Vitis vinifera*	China	KU140694	KU140676	KU140685
N. zimbabwana	CBS 111495	*Leucospermum cuneiforme*	Zimbabwe	JX556231	KM199545	KM199456
P. trachicarpicola	OP608	*Trachycarpus fortunei*	China	JQ845947	JQ845945	JQ845946
Pseudop. cocos	CBS 272.39	*Cocos nucifera*	Indonesia	KM199378	KM199553	KM199467

New sequences are set in bold font. Ex-type/ex-epitype isolates are marked by *.


86/91
CFCC 51992 N. rosicola
CFCC 51993 N. rosicola
CBS 367.54 N. aotearoa
64/91
CBS 394.48 N. piceana
CBS 225.30 N. piceana
65/82
CBS 225.32 N. piceana
MFLUCC 12-0233 N. samarangensis
100/100
MFLUCC 12-0261 N. clavispora
MFLUCC 12-0262 N. clavispora
MFLUCC 15-0152 N. cocois
100/100
MFLUCC 12-0283 N. ellipsospora
MFLUCC 12-0284 N. ellipsospora
CGMCC 3.9123 N. foedans
92/92
CGMCC 3.9202 N. foedans
CGMCC 3.9178 N. foedans
CBS 114178 N. protearum
CBS 257.31 N. javaensis
-/65
CBS 101057 N. rosae
-/51
CBS 124745 N. rosae
CBS 336.86 N. mesopotamica
MFLUCC 12-0282 N. saprophytica
MFLUCC 12-0286 N. asiatica
99/99
MFLUCC 12-0261 N. chrysea
56/73
84/98
MFLUCC 12-0285 N. umbrinospora
KT950861 Fungal Planet 372 N. egyptiaca
87/88
CBS 114159 N. australis
50/55
MFLUCC 15-126535 N. vitis
MFLUCC 15-0766 N. musae
CBS 264.37 N. eucalypticola
65/76
CBS 114495 N. honoluluana
98/92
CBS 111495 N. zimbabwana
100/100
CBS 600.96 N. cubana
CBS 115.83 N. formicidarum
64/67
CBS 362.72 N. formicidarum
CBS 450.74 N. surinamensis
81/82
MFLUCC 12-652 N. magna
100/99
CBS 138.41 N. natalensis
IMI 192475 N. steyaertii
CBS 272.29 Ps. cocos
OP068 P. trachicarpicola
20.0

Results

Molecular phylogeny

The combined ITS, TEF1, and TUB2 data matrix contains 38 ingroup and two outgroup isolates. The statistics for the MP analysis revealed that of 1461 characters, 1074 characters were constant, 214 variable characters were parsimony-uninformative, and 173 characters were parsimony-informative. The MP analysis yielded one most parsimonious tree, with the tree (TL = 650, CI = 0.737, RI = 0.702, RC = 0.517) shown in FIG. 1. The phylogenetic trees obtained from ML and BI analyses were consistent with the MP tree. A novel species was identified based on the multi-locus phylogeny coupled with the morphological features described below.

Pathogenicity

On stem wounds of the treatment group, lesions developed gradually and conidiomata were produced 10 days after inoculation, while few changes were observed in the control group (FIG. 2). Newly produced conidiomata sampled from the lesions were identified as *Neopestalotiopsis rosicola* by morphological observation of conidiogenous cells and conidia.

Taxonomy

Neopestalotiopsis rosicola C.M. Tian & Ning Jiang, **sp. nov.** FIG. 3

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Differs from *Neopestalotiopsis rosae* by its shorter and less numerous tubular apical appendages.

TYPE: China, Jiangsu Prov., Gaoyou City, 32°47′25″N 119°28′12″E, 1 m asl, on twigs and branches of *Rosa chinensis* Jacq., 12 Feb 2017, coll. N. Jiang (**Holotype,** BJFC-S1386; ex-type culture CFCC 51992; GenBank KY885239, KY885243, KY885245).

ETYMOLOGY: *rosicola* (Lat.), named after the host genus, *Rosa*.

ON PDA: CONIDIOMATAL PYCNIDIA globose to oval, solitary or confluent, semi-immersed, black, 100–550 µm diam; exuding globose, black, glistening, conidial masses. CONIDIOPHORES indistinct, often reduced to conidiogenous cells. CONIDIOGENOUS CELLS discrete, cylindrical to subcylindrical, hyaline, rugose-walled, 5–20 × 2–7 µm, apex 1–5 µm diam. CONIDIA fusoid, ellipsoid, straight to slightly curved, 4-septate, (18.9–)20.2–25.5(–26.2) ×

FIG. 1 (at left). Phylogram of *Neopestalotiopsis* based on combined ITS, TEF1, and TUB2 data. MP and ML bootstrap support values >50% are shown at the first and second position. Thickened branches represent BI posterior probabilities >0.90. Scale bar = 20 nucleotide substitutions. Ex-type strains are in bold and the new species is in blue.

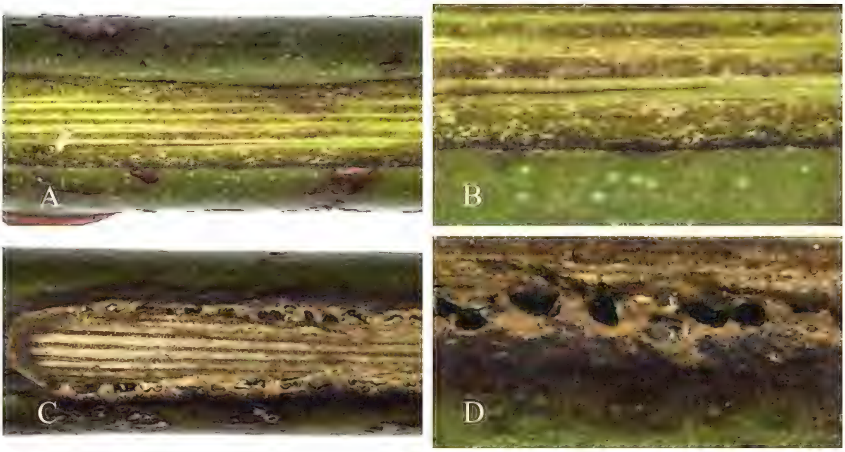

Fig. 2. *Neopestalotiopsis rosicola*: pathogenicity tests on *Rosa chinensis* stems. A, B: Control treatment wounds 10 days after being covered by pure PDA. C, D: Pathogen treatment wounds 10 days after inoculation, showing numerous sclerotia (and with conidiomata also formed).

(5–)5.5–8(–8.5) µm, mean ± SD = 22.9 ± 0.8 × 6.5 ± 0.5 µm; basal cell conic with a truncate base, hyaline, thin-walled, 2.9–5.8 µm long; three median cells doliiform, (10.7–)12.7–16.3(–18.6) µm long, mean ± SD = 10.5 ± 0.8 µm, wall rugose, versicoloured, septa darker than rest of cell; second cell from base pale brown to olivaceous, 3–6 µm long; third cell honey brown, 4.3–6.1 µm long; fourth cell brown, 3.4–6.5 µm long; apical cell 3–5 µm long, hyaline, cylindrical to subcylindrical, thin and smooth walled, with 2–4 tubular apical appendages (mostly 2 and 3, seldom 4), arising from apical crest, flexuous, unbranched, (16.5–)17–22.8(–25.9) µm long, mean ± SD = 20 ± 0.9 µm; basal appendage single, tubular, unbranched, centric, 2–9.5 µm long. Colonies on PDA attaining 40–70 mm diam after 7 days at 25°C, with undulate margin, whitish; after 15 days at 25°C, black conidiomata distributed irregularly on surface.

Host/Distribution: on branches of *Rosa chinensis* in China.

Additional specimen examined: **CHINA, Jiangsu Prov., Gaoyou City**, 32°47′25″N 119°28′12″E, 1 m asl, on twigs and branches of *Rosa chinensis* Jacq., 12 Feb 2017, coll. N. Jiang (BJFC-S1387; culture CFCC 51993; GenBank KY88240, KY885244, KY885246).

Note: The two isolates of *Neopestalotiopsis rosicola* cluster in one supported clade (MP/ML/BI = 86/91/1), closely related to *N. aotearoa* Maharachch. & al. isolated from canvas and *N. piceana* Maharachch. & al. on *Picea* sp. (Fig. 1). The new species differs from *N. aotearoa* by its longer and more

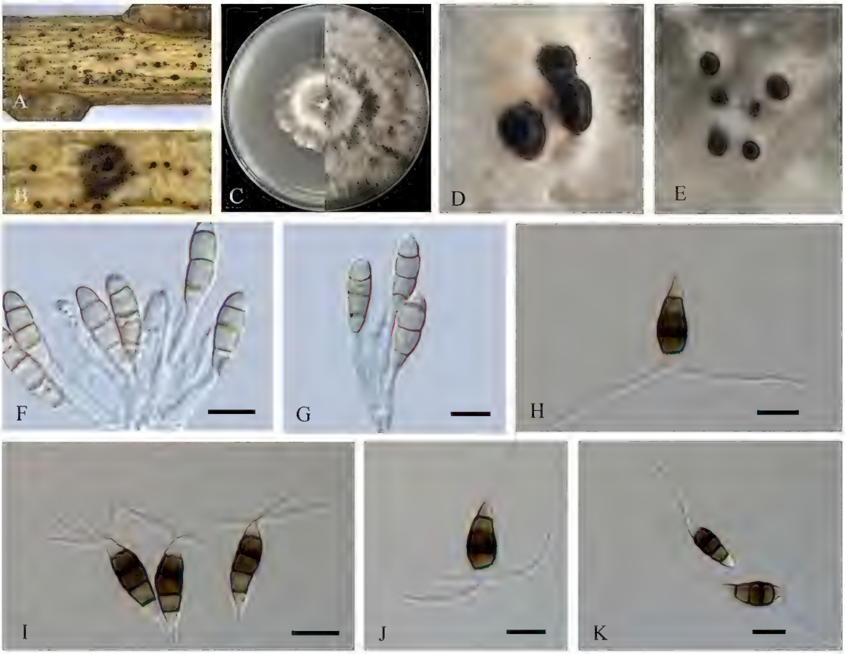

Fig. 3. *Neopestalotiopsis rosicola* (holotype, BJFC-S1386). A, B: Habit of conidiomata on *Rosa chinensis* stem. C: Colonies on PDA at 3 days (left) and 15 days (right). D, E: Conidiomata on PDA. F, G: Conidiogenous cells. H–K: Mature conidia. Scale bars: F–K = 10 µm.

numerous tubular apical appendages and from *N. piceana* by shorter and more numerous tubular apical appendages (Table 2).

Discussion

Neopestalotiopsis rosicola associated with a canker disease of *Rosa chinensis* in Jiangsu Province, China, is introduced as a new species based on morphological characteristics and molecular data (ITS, tef1, and tub2). It is distinguished from two *Neopestalotiopsis* species previously described from *Rosa*. *Neopestalotiopsis rosae* causing stem lesions on *Rosa* sp. in New Zealand (Maharachchikumbura & al. 2014) is morphologically distinct from *N. rosicola* (Fig. 1; Table 2) and produces only a few conidiomata on the diseased host tissue. *Neopestalotiopsis clavispora,* causing rose leaf blotch in China (Feng & al. 2014), differs from *N. rosicola* by its longer and less numerous tubular apical appendages (Table 2). The three species on *Rosa* clustered in separate clades in the combined *Neopestalotiopsis* phylogeny (Fig. 1).

TABLE 2. Comparison of *Neopestalotiopsis rosicola* and related species

SPECIES	STRAIN	CONIDIA (µm)	APICAL APPENDAGES		HOST/SUBSTRATE	LOCATION	REFERENCE
			NUMBER	LENGTH (µm)			
N. aotearoa	CBS 367.54	21–28 × 6.5–8.5	2–3	5–12	Canvas	New Zealand	Maharachchikumbura & al. 2014
N. clavispora	YJYK-1	18–26 × 6–8.5	2–3	19–32	*Rosa chinensis*	China	Maharachchikumbura & al. 2012; as *Pestalotiopsis clavispora*
N. piceana	CBS 394.48	19.5–25 × 7.5–9	3	21–31	*Picea* sp.	UK	Maharachchikumbura & al. 2014
N. rosae	CBS 101057	22–37 × 7.5–9.5	3–5	24–31	*Rosa* sp.	New Zealand	Maharachchikumbura & al. 2014
N. rosicola	**CFCC 61992**	**20.2–25.5 × 5.5–8**	**2–4**	**17–23**	***Rosa chinensis***	**China**	This paper

The new species is set in bold font.

Neopestalotiopsis rosicola was verified as the pathogen causing China rose canker disease in our pathogenicity test. However, preliminary experiments with mycelium from *N. rosicola* (data not shown) indicated that direct infection of the host is unlikely. Considering that these ornamental plants are regularly pruned, we inoculated artificially induced wounds. *Neopestalotiopsis rosicola* was shown to infect wounded hosts quickly, producing conidiomata in 10 days and capable of being re-isolated from the diseased host. We suggest that gardeners adopt protective measures during pruning, especially in Jiangsu Province.

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